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(54) Title: TELOMERE MIMETIC COMPOUNDS AND METHODS OF USE FOR SAME

(57) Abstract

A method of inhibiting proliferation of immortal cells or cells that express telomerase is disclosed. The method includes introduction of synthetic oligonucleotides which comprise telomere mimetic sequences. Applicant has identified specific oligonucleotides and characteristics which must be present for such oligonucleotides to increase cellular uptake and to maximize inhibition of proliferation as well as cytotoxicity.

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TITLE: TELOMERE MIMETIC COMPOUNDS AND METHODS OF USE FOR SAME

BACKGROUND OF THE INVENTION

Normal human somatic cells (e.g. fibroblasts endothelial and epithelial cells) display a replicative capacity of 50-200 population doublings characterized by a cessation in proliferation in spite of the presence of abundant growth factors. This cessation of replication *in vitro* is referred to as cellular senescence or cellular aging. The replicative life span of cells is inversely proportional to the *in vivo* age of the donor thus cellular senescence is suggested to play an important role in aging *in vivo*.

Cellular immortalization (the acquisition of unlimited replicative capacity) may be thought of as an abnormal escape from cellular senescence. Normal human somatic cells have finite replicative potential. In contrast germ line and malignant tumor cells are immortal (have indefinite proliferative potential). This has been postulated in part to occur via abnormal telomere function and the activation of telomerase.

A telomere is a specialized structure at the 3' end of chromosomes in most living organisms. It functions in chromosome protection, positioning, and replication. Generally, telomeres consist of hundreds to thousands of tandem repeats of a telomere motif sequence and associated proteins. In humans and other mammals this motif is 5'-d(TTAGGG)-3' . Sequences specific to other species such as yeasts, plants and ciliates may be found in Greider, Carrol, "Telomeres Telomerase and Senescence", Bio Assays, Vol. 12, No. 8, August 1990, pp. 363-369, incorporated herein by reference.

In humans the average telomere length of sperm DNA is 15 kb. As an organism ages, however, its telomere length becomes shorter. This phenomenon has caused telomeres to become characterized as a mitotic clock, responsible for the limited proliferative capacity of somatic cells. It has been demonstrated that chromosomes lose about 50-200 nucleotides of telomeric sequence per cell division, from the 3' end of the DNA molecule.

This loss of sequence from the 3' end of DNA is primarily due to the activity of DNA polymerase and its replication of DNA prior to cell division. DNA polymerase requires a primer for DNA replication and proceeds only in the 5' to 3' direction. The molecular end of the DNA molecule with the 3' end leading strand proceeds to the end of the DNA molecule, while lagging strand replication must utilize RNA primers and Okazaki fragments synthesis. Removal of the RNA primers Okazaki fragment ligation leaves a region at one end of each daughter molecule unreplicated. With no mechanism to fill the gap, the chromosome gets shorter with each round of replication. After numerous rounds of replication a great amount of DNA is lost from chromosome ends. Telomeres thus provide a measure by which a cell can gauge its divisions. A sufficiently short telomere is postulated to be the signal for replicative senescence, or apoptosis in normal cells.

In stark contrast, virtually all immortal cells and stem cells examined to date show no net loss of telomere length or sequence with cell division, suggesting that maintenance of telomeres is required for cells to escape from replicative senescence and to proliferate indefinitely. The enzyme capable of synthesizing telomere sequence is telomerase.

Telomerase is a ribonucleoprotein that synthesizes telomeric DNA onto 3' chromosome ends. Using a segment of its RNA component as a template, it can perform denovo synthesis of the telomeric repeat (5'-d(TTAGGG)-3' for humans and other mammals). This function thus compensates for the inability of DNA polymerase to completely replicate the ends of linear DNA molecules. Telomerase could also function to protect ends of linear chromosomes from degradation, recombination, and ligation to other chromosome ends. Telomerase activity has been identified in germ line cells and has been detected in 98% of immortal cell lines and in 95% of biopsied tumor tissue. The enzyme is generally not present in normal somatic tissues.

Induction of telomerase is postulated to be a key component in the progression of a pre-malignant condition to malignancy. Without wishing to be bound by any theory it is postulated that inhibition of telomerase in an

immortalized cell line or in the malignant condition would cause senescence or cell death if the cell depended on telomerase to compensate for loss of telomeric DNA through cell division.

The RNA moiety of human telomerase contains the 5'-CCCTAA-3' sequence that may act as the template for denovo synthesis. The enzyme also contains a region that recognizes the guanine rich single strands of DNA substrate.

Several genetic and molecular biology methodologies have been employed which take advantage of telomerase and the telomere motifs, for diagnosis of disease, for lengthening telomeres to increase cellular proliferation, or to inhibit immortalized cell proliferation through inhibition of telomerase.

For example, PCT publication No. WO 93/23572 discusses cellular senescence and immortalization via the activity of telomeres and telomerase. While the invention is primarily drawn to diagnostic methods of measuring telomere length in characterizing neoplastic cells, it discusses the concept of hybridization of oligonucleotides to telomeres or telomerase. The application specifically limits such oligos to at least two or more and not more than 50 telomere motif repeats (between 12 and 300 bases). Further when a cell line was treated with an oligonucleotide, 5'-d$(TTAGGG)_2$ -3' it required as many as 6 days in culture before any observable response was obtained. The application concludes that an oligonucleotide must be chosen which binds to telomerase at a different site than that bound by the (5'-d$(TTAGGG)_2$ -3') oligo for any desirable inhibition of replicative capacity.

PCT published document WO/9513383 discloses methods and compositions for increasing telomere length in normal cells to increase the proliferative capacity of cells and to delay the onset of cellular senescence. According to the disclosure, a telomere extending amount of an agent that increases telomere length within the cell is provided to a cell. This is accomplished by provision of an oligonucleotide that acts to lengthen telomeres in cell culture or cultivation thus increasing the number of cell divisions that

can occur. By virtue of the method, one can lengthen telomeres by providing nucleic acid sequences that can act as a telomerase substrates to the cells. Such nucleic acid sequence are disclosed to include at least two to three telomeric repeat sequences. The methods are disclosed for cell therapy which involves isolation of healthy human cells, expansion of those cells ex vivo and reinfusion of the expanded cells to a patient for treating such things as bone marrow therapy for hematological disorders such as anemia, leukemia and lymphoma.

PCT published document WO 95/13382 discloses methods and compositions for determination of telomere length and telomerase activity as well as the ability to increase or decrease telomerase activity in the treatment of proliferative diseases. According to the disclosure, primers are elongated under conditions which minimize interference from other genomic sequences so to obtain accurate determinations of telomeric length or telomerase activity. In addition, compositions are provided for intracellular inhibition of telomerase activity using a double telomere repeat 5'-TTAGGGTTAGGG-3' (SEQ ID NO:1).

United States Patent No. 5,643,890 to Iversen et al. discloses synthetic oligonucleotides which mimic telomeric sequences for treatment of cancer and other diseases. The patent discloses that a single telomere motif 5'-TTAGGG-3', when administered to immortal cells exhibited greater cellular uptake and higher inhibition of proliferation than longer oligonucleotides which were suggested in the art and which were thought to be necessary to achieve appropriate sequence specific interaction.

There is a continuing need in the art for more refinement of methodologies using telomeric sequences as therapeutic agents for inhibition of cellular proliferation and killing of immortalized cells.

It is an object of the present invention to provide telomere mimetic oligonucleotides which are optimized for telomerase inhibition, cellular uptake, sequence specific interaction, and cell killing of neoplastic cells.

It is a further object of the present invention to provide pharmaceutical compositions which contain optimized telomere mimetic sequences for administration to cells for treatment of cancer and other diseases characterized by uncontrolled cell proliferation.

It is a further object of the present invention to provide pharmaceutical compositions comprising optimized telomere mimetic compounds in combination with other pharmaceutical agents which potentiate the cytotoxic effects of administered oligonucleotides.

Further objects will become apparent from the detailed description of the invention which follows.

SUMMARY OF THE INVENTION

The present invention relates to oligonucleotide strategies which are designed to forestall the capacity of neoplastic cells to uncontrollably proliferate. The strategy specifically targets naturally occurring biochemical agents in neoplastic cells which allow for their escape from cellular senescence and inhibits these agents to cause cell death.

According to the teachings herein, a method of anti-neoplastic therapy through activity of proliferation inhibitors (oligonucleotides) is disclosed. According to traditional oligonucleotide therapy, a synthetic oligonucleotide of approximately 14 nucleotides, preferably complementary to DNA (antigen) or RNA (antisense), which interferes with the process of transcription or translation of endogenous proteins in cells or in animals is introduced. Applicants have discovered that oligonucleotides of less than 12 bases preferably as few as 6-9 bases which are designed to mimic or interact with telomere motifs cause a significant decrease in immortal cell proliferation by inhibiting telomerase expression and also causing immortal cell death, through a second mechanism, likely by steric hindrance apart from telomerase inhibition. Longer oligonucleotides may also be increased in cytotoxicity through the prevention of guanine secondary structure formation, rather than use of short oligonucleotide length. Telomere mimicking sequences which

retain the guanine triplet motif, (TTAGGG)or which employ multiple guanine triplets or even which use further addition of guanine bases have been shown to most potent for cytotoxicity.

As used herein, the term telomere motif shall mean a 6-mer sequence which employs a guanine triplet.

As used herein the term "antisense" is intended to encompass all forms of oligonucleotide strategies discussed herein which inhibit telomerase or which result in immortal cell killing based upon the telomere motifs disclosed herein, whether anti-gene antisense or telomere decoy sequences.

DESCRIPTION OF THE FIGURES

Figure 1 is the graph depicting the effect of glyoxalation of TAG 9-2 for cell viability as measured spectrophotometrically. As can be seen, the TAG 9-2 demonstrates the highest cell toxicity while glyoxalated TAG 9-2 demonstrates significantly decreased cytotoxicity. (A= untreated; B= TAG 9-2; and C= TAG 9-2 Glyoxal)

Figure 2 is graph depicting the effect of adding glycerol to the 3' end of TAG 9-2 on cell viability using the identical assay of Figure 1. As can be seen the addition of glycerol decreases the cytotoxicity of TAG 9-2 (A= TAG 9-2; B= TAG 9-2 3' Glycerol; and C= Untreated Cells).

Figure 3 is a graph depicting the cell viability effects of 7-deaza-G incorporation on TAG 18 (A= TAG 18; B= Untreated Cells; and C= TAG 18 7-deaza-G). The deaza treated oligo which prevents guanine secondary pairing greatly increased cytotoxicity of the telomere compound.

Figure 4 is a graph depicting cell viability and the effect of adding a G 5' to TAG 9-2 (A= TAG 9-2; B= TAG 9-2 5'G; and C= untreated cells). The addition of the 5'G to TAG 9-2 increased cytotoxicity of the telomere compound at lower concentrations.

Figure 5 is a bar graph depicting the effect of randomizing base numbers 1-6 in TAG 9-2 and the effect on cell viability. As can be seen, bases 4, 5, and 6 had little effect on cytotoxicity compared to that of TAG 9-2 while

randomizing bases 1, 2, and 3 greatly decreased cytotoxicity of the telomere compound.

Figure 6 is a graph depicting the effect of adding a spacer to TAG 9-2 (A= 9-2~; B= TAG 9-2; and C= untreated cells). As can be seen, the spacer increased the cytotoxicity of TAG 9-2.

Figure 7 is a graph depicting the effect of an Abasic site spacer on TAG 9-2 (A= TAG 9-2~; B= TAG 9-2^; and C= untreated cells). Both spacers caused a decrease in cell viability compared to untreated cells.

Figure 8 is a graph depicting the relationship between cytotoxicity and telomerase inhibition (A= 9-2^; B= 9-2^ 5'G; C= 9-2~; D= 9-2; E= 9-2glyc; and F= 9-1).

Figure 9 is a bar graph depicting the effect of free radical scavengers on telomere mimic induced cytotoxicity (A= 1μM ascorbate; and B= untreated).

Figure 10 is a graph depicting the amount of DNA present in the media after treatment with sodium nitroprusside, an oxygen radical generator (A= 25mM; and B= 12.5mM).

Figure 11 is a graph depicting the treatment of Oma-BL1 cells with 12.5mM sodium nitroprusside, in the presence of a telomere compound and with no oligonucleotide.

DETAILED DESCRIPTION OF THE INVENTION

Antisense oligonucleotides represent potential tools in research and therapy by virtue of their ability to specifically inhibit synthesis of target proteins. A major theoretical advantage of these oligos is their potential specificity for binding to one site in the cell. According to the invention the oligo is introduced to cells by methods which are known to those of skill in the art. See, Iversen, et al., "Anti-Cancer Drug Design", 1991, 6531-6538, incorporated herein by reference. While not wishing to be bound by any theory, it is postulated that hybridization of proteolytic enzyme mRNA with the introduced complementary oligo occurs, effectively blocking translation.. The nucleotide sequences of the modified oligos which inhibit synthesis of

these enzymes need not be wholly (100%) complementary to be useful in the present invention. As used herein the term "substantially analogous" shall mean an oligo of approximately 80% complementarity or homology to the telomere sequence or repeat motif.

Aptamers are synthetic chains of nucleotides that block the disease-causing activity of certain proteins. For example, the thrombin-inhibitor GS522, a synthetic oligonucleotide designed as an anti-coagulant that binds thrombin (Gilead Sciences, Annual Report, 1994 p. 16-17).

The present invention employs a single-stranded synthetic oligonucleotide as a telomere motif decoy which effectively inhibits the telomerase enzyme and which also through a second mechanism, kills immortalized cells. The inventors have identified several critical parameters which should be present in such oligonucleotides to maximize the inhibition of telomerase, the decrease in cell proliferation, and the cell killing accomplished by telomeric antisense therapy.

The advantages of such a strategy include fewer competing sites (there are less than approximately 50 endogenous sites in the human genome), inhibition of telomerase activity involves both hybridization of complementary sequences of nucleic acids (antisense) and allosteric binding (aptameric binding) to the ribonucleoprotein complex, the telomere which the oligonucleotide mimics is identical in all higher vertebrates, hence drug development can utilize animal models, and the ease and cost in purification of shorter oligonucleotides disclosed herein is considerably less than a standard antisense oligo.

Also disclosed is combination therapies of antisense oligonucleotides combined with other pharmaceutical agents which potentiate the effects of the telomere mimics.

Traditional limitations of oligonucleotide therapy have been preparation of the oligonucleotide analogue which is substantially resistant to the endo and exonucleases found in the blood and cells of the body. While unmodified

oligos have been shown to be effective, several modifications to these oligos have helped alleviate this problem.

Modified or related nucleotides of the present invention can include one or more modifications of the nucleic acid bases, sugar moieties, internucleoside phosphate linkages, or combinations of modifications at these sites. The internucleoside phosphate linkages can be phosphorothioate, phosphoramidate; methylphosphonate, phosphorodithioate and combinations of such similar linkages (to produce mixed backbone modified oligonucleotides). Modifications may be internal or at the end(s) of the oligonucleotide molecule and can include additions to the molecule of the internucleoside phosphate linkages, such as cholesterol, diamine compounds with varying numbers of carbon residues between the amino groups, and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the genome. While oligonucleotides have been shown to be effective without any modifications, these modifications traditionally help shield it from enzymatic degradation within the cell.

Pharmacokinetics of phosphorothioate oligonucleotides have been studied a great deal and have been shown to be favorable for *in vivo* treatment methods. See "Cellular Uptake In Subcellular Distribution Of Phosphorothioate Oligonucleotides Into Cultured Cells", Antisense Research & Development II: 211-222 (1992). Iversen et al. incorporated herein by reference which demonstrates that phosphorothioate oligonucleotides are taken up into cells within one hour and sequestered into both nuclei and mitochondria of cells in a time-dependent manner. Also incorporated herein by reference is the article "*In Vivo* Studies With Phosphorothioate Oligonucleotides: Pharmacokinetics Prologue, Iversen, P. Anti Cancer Drug Design (1991), 6531-538. This paper discusses the half-life of phosphorothioate oligonucleotides administered intravenously or intra peritoneally and discloses a biphasic plasma elimination of the oligonucleotide including a distribution half-life of 15-25 minutes and an elimination half-life

of 20-40 hours and that repeated in daily injections of oligonucleotide provides steady state concentration in 6-9 days and indicates that phosphorothioate oligos are relatively non-toxic and concludes "pharmacokinetic considerations are not likely to be limiting factors in anti-cancer drug design with phosphorothioate oligonucleotides.

Also incorporated by reference herein are "Iversen et al, Pharmacokinetics of an anti-sense phosphorothioate oligo oxynucleotide against *rev* and from human immunodeficiency virus Type I in the adult male rat following single injections and continuous infusion, Antisense Research & Development, 4, 43-52 (1994). Again, this article discusses uptake and distribution of phosphorothioate oligonucleotides administered through intravenous injections and subcutaneous infusions. No toxicity was seen and plasma concentration was directly proportional to dose. Oligonucleotide was completely eliminated in urine over 3 days again concluding that phosphorothioate oligonucleotides are promising as therapeutic agents *in vivo*. Further Agrawal et al. 1991 indicate that phosphorothioate oligonucleotides can achieve potentially therapeutic concentrations (about 0.1-1 μM in at least several tissues of mice, rats and monkeys. Degradation is slow *in vivo*. Further incorporated hereby in reference is Bayever et al, Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for acute myelogenous leukemia and myelodysplastic syndrome: initial results of a Phase I trial. Antisense Research and Development 3:383-390 (1993). This paper discusses pharmacokinetics of phosphorothioate oligonucleotides administered to human patients. Oligonucleotide was administered via continuous intravenous infusion and no major toxicity was seen for a dose of 0.05 mg/kg/hr. The article concludes that safety and favorable pharmacokinetics support the further investigations of phosphorothioate oligonucleotides as potential gene specific therapeutic agents in humans. Finally applicant hereby incorporates by reference "Oligonucleotides as Therapeutic Agents", Ciba Foundation Symposium, 209,

1997 John Wiley and Sons, West Sussex England, which documents similar favorable pharmacokinetic parameters of oligonucleotide base pharmaceutical agents.

Any of the known methods for oligonucleotide synthesis can be used to prepare the oligonucleotides. They are most conveniently prepared using any of the commercially available, automated nucleic acid synthesizers, such as Applied Biosystems, Inc., DNA synthesizer (Model 380B), According to manufacturers protocols using phosphoroamidite chemistry. Phosphorothioate oligonucleotides were synthesized and purified according to the methods described in Stec and Zon J. Chromatography, 326:263-280 and in Applied Biosystems, DNA Synthesizer, User Bulletin, Models 380A-380B-381A-391-EP, December 1989.

As used herein the term "therapeutically effective amount" of an oligonucleotide is a well recognized phrase. Those skilled in the medical arts will readily appreciate that the doses and schedules of oligonucleotide will vary depending on the age, health, sex, size and weight of the patient rather than administration, etc. These parameters can be determined for each system by well-established procedures and analysis e.g., in phase I, II and III clinical trials. Dosing schedules, pharmacokinetics and toxicity of oligonucleotide therapeutics are discussed in detail in "Oligonucleotides as Therapeutic Agents", previously incorporated herein.

For human *in vivo* use preferred dosage of the oligonucleotides of the present invention is that which is necessary to attain a concentration in blood of from about 0.01 to about 1 μM. This concentration can be achieved in a variety of ways. Doses of between about 0.05 and about 0.25 mg/kg/hr by continuous IV infusion have been found to be acceptable. Greater or lesser amounts of oligonucleotide may be administered as required.

For such administration the oligonucleotide or conjugate thereof can be combined with a pharmaceutically acceptable carrier such as a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid

vehicles and excipients are conventional and are commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose and the like. Traditionally IV therapy is preferred.

In general, in addition to the active compounds, the pharmaceutical compositions of this invention may contain suitable excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Oral dosage forms encompass tablets, dragées, and capsules. Preparations which can be administered rectally include suppositories. Other dosage forms include suitable solutions for administration parenterally or orally, and compositions which can be administered buccally or sublingually.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself well known in the art. For example the pharmaceutical preparations may be made by means of conventional mixing, granulating, dragée-making, dissolving, lyophilizing processes. The processes to be used will depend ultimately on the physical properties of the active ingredient used.

Suitable excipients are, in particular, fillers such as sugars for example, lactose or sucrose mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch, paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added, such as the above-mentioned starches as well as carboxymethyl starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are flow-regulating agents and lubricants, for example, such as silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate and/or polyethylene glycol. Dragée cores may be

provided with suitable coatings which, if desired, may be resistant to gastric juices.

For this purpose concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate, dyestuffs and pigments may be added to the tablet of dragée coatings, for example, for identification or in order to characterize different combination of compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition stabilizers may be added. Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of the active compounds with the suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffinhydrocarbons, polyethylene glycols, or higher alkanols. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base material include for example liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of active compounds in water-soluble or water-dispersible form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles

include fatty oils for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, include for example, sodium carboxymethyl cellulose, sorbitol and/or dextran, optionally the suspension may also contain stabilizers.

In addition to administration with conventional carriers, active ingredients may be administered by a variety of specialized delivery drug techniques which are known to those of skill in the art.

Applicants have discovered a synthetic oligonucleotide which inhibits telomerase and leads to cell death in neoplastic cells. In one aspect the invention contemplates a method for treating conditions or diseases *in vitro* or *in vivo* which are related to the ability of a cell to remain immortal and to proliferate uncontrollably. Examples of such cells include cancerous cells which are somatic cells which have regained the ability to express telomerase, primarily associated with treatment of cancers of any type including solid tumors and leukemias including those in which cells are immortalized such as apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., b-cell, mixed-cell, null-cell, T-cell, T-cell chronic, HTLV-II-associated, lyphocytic acute, lymphocytic chronic, mast-cell, and myeloid), histiocytosis malignant, Hodgkin's disease, immunoproliferative small, non-Hodgkin's lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing's sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma,

throphoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, leydig cell tumor, papilloma, sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, antiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing's experimental, Kaposi's, and mast-cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia), and for treatment of other conditions in which cells have increased proliferation. For example, HIV infected animals exhibit increased proliferation of CD4+ cells. The invention could thus be used to quell this increased proliferation. Those of skill in the art will readily appreciate that other diseases so characterized can be treated by the method of the invention.

Cells or disease states more specifically treatable by the method of the invention can be identified by an *in vitro* assay of the cells for telomerase activity. There are numerous assays available in the art to determine the presence of telomerase in a particular cell line or tumor. Tumor cells *in vivo* may be simply removed and tested for presence of telomerase or the *in vitro* cell line may be used. One such assay is listed in Science, Vol. 266, December 23, 1994, Nam W. Kim, et al., "Specific Association of Human Telomerase

Activity With Immortal Cells and Cancer", pp. 2011-2014. See also Prowse, Karen R. et al., "Identification of a nonprosessive telomerase activity from mouse cells" P.N.A.S. Vol 90 Pp. 1493-1497, Feb. 1993. Generally, assay methods include hypotonic swelling and physical disruption of cells requiring 10^7 to 10^8 cells and extraction by detergent lysis. A more sensitive recently developed assay includes PCR based assay wherein telomerase synthesized extension products serve as templates for PCR amplification which can be performed in a single reaction.

Applicants have discovered that oligonucleotides, shorter nucleotides or ones which are prohibited from forming secondary guanine pairings, preferably of 6-9 bases exhibit marked decrease in tumor cell proliferation over other oligonucleotides with multiple telomere motifs. Oligonucleotides which are modified to prevent secondary guanine pairing are known in the art and described in "Oligonucleotides as Therapeutic Agents", supra. These include but are not limited to deaza modification, short (generally 12 or less bases) oligo and length. This is an unexpected result as traditional oligonucleotide therapy postulates stable hybridization of the oligonucleotide to endogenous target sequences. It is generally thought that at least at physiological salt and at 37°C oligonucleotides of less than 12 bases will not form stable hybrid duplexes. Oligonucleotides of this short length will not form stable hybrid duplexes at 37°C possibly indicating a non-antisense type mechanism contributes to telomerase inhibition.

According to the invention, Applicants have identified several important features for maximizing the effectiveness of administered telomere mimetic compounds.

Applicants previously discovered that an extremely short oligonucleotide 5'-d(TTAGGG)-3' will inhibit telomerase with observable S-phase delay in the cell cycle and will result in a dramatic increase in apoptotic cell death.

Applicants had found that the telomere sequence phosphorothioate-modified single repeat 6-mer 5'-d(TTAGGG)-3' in fact, can work better than

longer oligos with more repeats. While not wishing to be bound by any theory it is postulated that efficacy of oligonucleotide inhibition is length-dependent with regard to uptake and ultimately elimination of immortal phenotype. Such shorter oligonucleotides are less expensive, easier to synthesize, and less likely to cause toxicity.

Applicants discovered that the guanine triplet of the telomere motif 5'-d(TTAGGG)-3' is of critical importance to inhibition of telomerase and to the efficacy of the inhibition of cell proliferation. Also the preference of a guanine on the 3' end has also been established. As such, short oligonucleotides, less than 12 bases or which are otherwise prevented from forming guanine secondary structures which mimic telomeric sequences and which optimize the numbers of guanine triplets are potent inhibitors of telomerase and cell proliferation. Applicants have designed an oligo with the telomere motif having two guanine triplets one 3' and one 5', 5'-d(GGGTTAGGG)-3' which was shown to have the highest efficacy of several compounds tested. This compound (named TAG 9-2) retains the benefits of a shorter oligonucleotide sequence yet also has two guanine triplets for interacting with native cellular sequences. The 3' end has been delineated of primary importance in achieving the decrease in cellular proliferation as steric blocking of the 3' end decreased efficacy of the compound.

Also critical to maintaining optimum efficacy is the ability of guanine present in the oligonucleotide to be able to hydrogen bond, glyoxalated TAG 9-2 decreased the efficacy of the oligo. Applicants have further identified that the TTA region between the two guanine triplets is of little importance to achieve efficacy. In fact when the TTA regions were randomized, efficacy remained essentially constant for the TAG 9-2 oligomer.

Finally Applicants also discovered that addition of one additional guanine to the TAG 9-2 molecule resulted in an increase in efficacy.

According to the invention a telomere mimicking compound to be optimized for inhibiting cell proliferation and to further induce cell killing should be less than 12 bases long or otherwise inhibited from forming

secondary structures, should have a 3' guanine and the 3' end must not be sterically blocked and should conserve at least the guanine triplet region of a telomere motif. Preferably the oligo will contain greater than 50% guanine with multiple guanine triplets or additional guanine residues apart from the telomere motif wherein X is any nucleotide base. Longer sequences, such as $(XXXGGG)_3$ may be optimized by the use of agents such as deaza modification to increase efficacy.

According to the invention the following represent examples of oligonucleotide sequences which would be optimal:

3'-GXXXGGG-5'

3'-GGXXXGGG-5'

3'-GGGXXXGGG-5' (TAG 9-2)

3'-GGGGXXXGGG-5' (SEQ ID NO:11)

3'-GGGXXXGGGG-5' (SEQ ID NO:2)

3'-GGGXXXGGGXXG-5' (SEQ ID NO:3)

3'-GGGXXXGGGXG-5' (SEQ ID NO:4)

3'-GXXGGGXXXGGG-5' (SEQ ID NO:5)

3'-GXGGGXXXGGG-5' (SEQ ID NO:6)

One of skill in the art will readily appreciate that other sequences may be generated and assayed for their cytotoxic and telomerase inhibiting effects based upon the teachings herein and are intended to be encompassed within the scope of this invention.

In a further embodiment applicants have discovered that free radical generators such as nitric oxide potentiate the cytotoxic effects of active telomere mimetic compounds.

Conversely free radical scavengers inhibit the effects of active telomere mimetic compounds. Thus the oligonucleotides of the invention in one embodiment are administered in combination with a free radical generator. These types of compounds are well known in the art.

For example, the generation of reactive radical species has been found to be involved in the cytotoxic effects of ionizing radiation (see, e.g., Petkau, Acta. Physiol. Scand. Suppl. (1980) 492:81-90 and Biaglow et al., Radiat. Res. (1983) 95:437-455), various chemotherapeutic agents (see, e.g., Tomasz, Chem. Biol. Interact. (1976) 13:89-97, Lown and Sim, Biochem. Biophys. Res. Commun. (1977) 77:1150-1157 and Borek and Troll, Proc. Natl. Acad. Sci. USA (1983) 80:1304-1307), and a variety of other biological processes, including aging, and the initiation and promotion stages of experimental carcinogenesis (see, e.g., DiGuiseppi and Fridovich, CRC Crit. Rev. Toxicol. (1984) 12:315-342 and Slater, Biochem. J. (1984) 222:1-15). The generation and release of reactive free radicals in the respiratory burst phenomenon used by various cells of the immune system is a well known mechanism of foreign target destruction. See, e.g., Bus and Gibson in Rev. Biochem. Toxicol., eds. Hodgson et al. (Elsevier, North Holland, 1979), pp. 125-149 and Badwey and Karnovsky, Ann. Rev. Biochem. (1980) 49:695-726.

As used herein, the term "free radical generation" refers to any cellular, tissue or other damage to body parts or functions sustained by the host as a result of free radicals being produced in the body of the host. The free radicals may cause directly mobilization of the arachidonic acid metabolic pathways or may cause lipid peroxidation that mobilizes arachidonic acid. These radicals may be produced as a mechanism for killing cells. Examples by which such damage may be caused include hyperthermia, which may occur during cancer treatment as when the temperature of the tumor is increased via local or general microwave irradiation, damage caused by chemotherapeutic agents (chemotherapy), radiation therapy, or high oxygen tension that produce radicals to kill cells, and infection. Also, treated tumor cells may help propagate radical damage. An example of high oxygen tension is the condition that occurs when premature babies are exposed to high pressure oxygen, resulting in retinal and lung disease. Other conditions that represent damage caused by free radical generation may also be envisioned and fall within this definition.

Cancer cells, particularly those which are malignant, exhibit elevated levels of free hydroxy radicals. These and other types of diseased cells do not exhibit the same degree of anti-oxidant protection as do normal cells. In particular, such cells are notably deficient in scavenger protection systems. Because of this fact, irradiation kills cancer cells preferentially to normal tissue and conventional radiation therapy attempts to exploit this mechanism of action, as do certain conventional chemotherapeutic agents, such as the nitrosoureas, e.g., BCNU (bischloroethylnitrosourea), and the anthracycline cytotoxic antibiotics, doxorubicin and daunorubicin.

Such conditions are produced, for example, by exposure of the cancer cell to radiation, or an agent capable of radical oxygen induced cytotoxicity, such as for example, anthracycline cytotoxic antibiotics (e.g., doxorubicin), BCNU, BSO (buthionine sulfoxamine), hydrogen peroxide, or antisense oligonucleotide inhibitors of SOD (superoxide dismutase), catalase, GSH synthetase, GSH reductase, or GSH peroxidase.

Administration of such agents are discussed in detail in United States Patent 5,641,754 the disclosure of which is incorporated herein by reference.

Further embodiments of the invention will be illustrated from the examples which follow and which are intended in no way to limit the scope of the invention. Those of skill in the art will readily appreciate that certain variations can be made to design other oligonucleotides according to the invention and are intended to be within the scope of the invention as described and defined herein.

EXAMPLE 1

Materials and Methods
Cell Culture

OMA-BL1 cells were obtained. The cells were cultured in RPMI-1640 media (Sigma, St. Louis, MO), supplemented with 10% heat inactivated fetal bovine serum (Gibco, Grand Island, NY), penicillin G (50 units/ml) and streptomycin (50μg/ml)(Sigma). The cells were subcultured at 3-4 day intervals to a density of 1 x 10^6 cells/ml in a 75 cm^2 flask containing 30ml of

medium, and incubated at 37°C in a humidified atmosphere of 95% air, 5% Co_2.

Oligonucleotide Synthesis and Purification

The ODNs were synthesized on a 1μmole scale by use of an Applied Biosystems Model 391 DNA Synthesizer (Foster City, CA). The phosphorothioate ODNs were synthesized and purified according to standard methodology known in the art. The 3' Glycerol ODN was synthesized using a commercially available CPG column with the glycerol molecule preattached (Glen Research, Sterling, WV). The 7-deaza Guanine 18mer was synthesized using a 7 deaza Guanine phosphoroamidite (Glen Research, Sterling, WV). The TAG9-2sp. and TAG9-1sp. molecules were synthesized using a spacer 9 phosphoramidite (Glen Research, Sterling, WV). The glyoxalated TAG9-2 and TAG9-1 was glyoxalated according to the procedures known in the art. It was verified using spectrofluoroscopy. The randomer S-ODNs were synthesized by mixing equimolar amounts of all 4 nucleotides together and using that bottle as the nucleotide for the base that we chose to randomize.

Cell Viability

The cell viability assay was based on the reduction of MTT (3,4,5 dimethylthiazol 2,5-diphenyl tetrazoluim bromide, Sigma) by mitochondrial dehydrogenase in viable cells to a formazan product that can be measured spectrophotometrically. OMA-BL-1 cells (300 cells in 100μl of media) were added to wells on a 96 well plate. 10μl of media containing ODNs of .3,1,3,and 10μmole concentrations was added to the wells. The plates were incubated for 72 hours. After 72 hours, the cells were lysed using .04N HCL in isopropanol and the absorbance was measured at 540 nm.

Trap Assay

The TRAP assays were performed using the TRAPeze Telomerase Detection Kit (ONCOR). Cell lysates, containing telomerase were harvested from OMA-BL-1 cells. The lysates were flash frozen in liquid nitrogen and stored at -70 C until used. 2μl of lysate were used for each TRAP reaction

containing 3ng protein/μl. 2μl of lysate plus 3μl of S-ODN were placed in each tube. 45 μl of the TRAP assay PCR mix were then added to each tube. The tubes were incubated for 30 minutes at 30°C. Two step PCR was then performed on the tubes 94°C/40s, 60°C/30s for 30 cycles). The PCR products were then run on a 12.5% non-denaturing PAGE in 1XTE Buffer. The gels were run for 3 hours at 300v. The gels were then fixed in 0.5M NaCl, 50% ethanol, 40mM sodium acetate (ph5.5) for 25 minutes. They were then exposed without drying to phosphor storage screens, they were visualized on a PhosphorImager using the ImageQuant software.

To determine the length requirements of a TAG sequence, a matrix of compounds was synthesized and tested on OMA-BL-1 cells. It was found that the longer TAG sequences had lower EC_{50} values than the shorter compounds overall (Table 1). Previous research had shown that having the guanine motif present on the 3' end was also important in the efficacy of these molecules.

TABLE 1
DETERMINATION OF LENGTH REQUIREMENTS

Sequence (5'-3')	EC_{50}
G	13.36 ± 2.161
GG	12.14 ± 1.955
GGG	15.59 ± 3.120
GGGG	2.827 ± .181
TTAG	≥ 20
TTAGG	≥ 20
TTAGGG	2.00 ± 0.20
TTAGGGT	≥ 20
TTAGGGTT	≥ 20
TTAGGGTTA	12.13 ± 1.612
GGGTTAGGG	2.936 ± .216
TTAGGGTTAG (SEQ ID NO:7)	6.54 ± 3.957
TTAGGGTTAGG (SEQ ID NO:8)	13.21 ± 1.691
TTAGGGTTAGGGTTAGGG (SEQ ID NO:9)	1.615 ± .214
TTAGGGTTAGGGTTAGGGTTAGGG (SEQ ID NO:10)	.22 ± 0.00

The guanine motif present on the 3' end was also found to be important in the efficacy of these molecules. Based on this a 9-base TAG sequence was synthesized so that the guanine motif was on the 3' end, 5'-d(GGGTTAGGG)-3'. The effect of this "frame shift" was to greatly decrease the EC_{50} of the 9-base TAG sequence. This shift placed the EC_{50} of the 9-base compound almost as low as that of the much longer TAG sequences.

Based on the results of Table 1 all possible reading frames of the 9-base telomere sequence motif were synthesized and again tested for anti-proliferative effects on OMA-BL-1 cells (Table 2).

Table 2
READING FRAME SHIFTS

Sequence (5'-3')	$EC_{50}\mu M$
TTAGGGTTA	12.13 ± 1.612
TAGGGTTAG	12.70 ± 1.627
AGGGTTAGG	4.36 ± .592
GGGTTAGGG	2.93 ± .217
GGTTAGGGT	13.75 ± 2.970

These experiments showed that the 5'-d(GGGTTAGGG)-3' exhibited the greatest efficacy. We call this compound TAG 9-2. These results again showed the importance of having a guanine nucleotide present at the 3' end of the TAG S-ODNs on their efficacy in OMA-BL-1 cell assay.

The role played by hydrogen bonding on the efficacy of TAG 9-2 was examined next. To block hydrogen bonding the TAG 9-2 S-ODN was glyoxalated(X). The glyoxalation reaction produces an etheno on guanine nucleotides that effectively blocks hydrogen bonding. While the yield of the reaction is not 100%, the etheno group formation was verified by spectroflouroscopy. The etheno group has distinct excitation and emission spectra that allows one to verify its presence. When OMA-GL-1 cells were treated with the glyoxalated TAG 9-2 compound, an appreciable decrease in the efficacy of the compound was observed, most notably at the 10X M concentration. Cells were also treated with TAG 9-1 and glyoxalated TAG 9-1, 5'-d(TTAGGGTTA)-3', this sequence does not decrease the viability of OMA-

BL-1 cells, yet is still a telomere sequence motif. This sequence was used throughout these studies as a negative control. In this experiment the TAG 9-1 S-ODNs had no effect. The conclusion drawn from the results of this experiment is that hydrogen bonding does play a role in the anti- proliferative action of TAG 9-2. See Figure 1.

Since telomerase is known to add repeats onto the 3' ends of chromosomes it was hypothesized that if TAG 9-2 was acting as a substrate for the telomerase enzyme, sterically blocking the 3' end of the molecule would decrease the efficacy. To test this hypothesis TAG 9-2 was synthesized with a glycerol molecule on the 3' end. The viability of OMA-BL-1 cells treated with the TAG 9-2G compound was increased when compared with the normal TAG 9-2 S-ODN, again, this effect was most notable at higher concentrations. No effect was observed on cells treated with TAG 9-1 or TAG 9-1G (Figure 2). The results of this experiment indicates that having a free 3' end is important in the mechanism of the cytotoxic effects of these molecules, it also lends support to the hypothesis that TAG 9-2 is interacting with the telomerase enzyme.

Guanine motifs in nucleic acids can form secondary structures, g-quartet and g-wire, in addition to the double helix. These secondary structures can be the result of intra and interstrand associations. The possibility exists that the longer TAG sequences can form these associations and that these associations might prohibit the TAG compounds from interacting with the telomerase enzyme. To test this hypothesis two guanine nucleotides that contain a deaza group at the G-7 position were incorporated into the TAG 18 S-ODN during synthesis. The location of the deaza group (G-7) sterically blocks the formation of the secondary structures. The TAG 18 S-ODN was used for these experiments because the longer TAG sequences presumably have a higher propensity to form the secondary structures. When OMA-BL-1 cells were treated with the TAG 18 7-deaza-G compounds an increased cytotoxic effect was observed than that for cells treated with the normal TAG 18 (Figure 3). By blocking the formation of secondary guanine structures the efficacy of TAG 18 was increased. Thus secondary structure

formation by the TAG S-ODNs decreases efficacy on OMA-BL-1 cells. These results support that telomerase as the enzyme is not able to interact with TAG molecules involved in the g-quarter or g-wire structures.

To optimize the efficacy of the TAG 9-2 S-ODNs a series of compounds was synthesized that contained one, or two extra guanine nucleotides, or thymine nucleotides on the 5' or 3' end of TAG 9-2. Most of these modifications did not improve the efficacy of the molecule, in fact many of them decreased it. However, the addition of one guanine nucleotide 5' to TAG 9-2 increased the efficacy of TAG 9-2 and shifted the dose response curve to the left (Figure 4).

To determine the importance of each base in the TAG 9-2 S-ODN a randomization study was done. Six S-ODNs were synthesized, each having one base randomized. The randomization was carried out by mixing equimolar amounts of all four nucleotides. This mix was then used to incorporate a random base at the specified position during the synthesis of these compounds. Only the first six bases were randomized because the importance of the guanine motif at the 3' end had already been established by the previous experiments. When OMA-BL-1 cells were treated with these compounds, a clear pattern emerged. See Figure 5. When bases one, two, or three were randomized the viability of the cells went up. These randomized compounds were much less efficacious than the normal, unrandomized, TAG 9-2. When bases four, five, or six, were randomized the viability of the cells was not different from the viability of the cells when treated with normal TAG 9-2, the efficacy of these randomized TAG S-ODNs was the same as that of the normal TAG 9-2. These results suggest that the cytotoxic effect observed from treatment with TAG 9-2 is not dependent on the sequence of the middle three nucleotides, the TTA portion of the S-ODN. However the cytotoxic effect is abrogated when bases one, two, or three, are randomized, suggesting that in these positions the correct sequence is important.

Based on the results of the previous experiment it was hypothesized that bases four, five, and six, the TTA portion of TAG 9-2, could be replaced with a spacer molecule, without decreasing its efficacy. The TTA portion of

TAG 9-2 was replaced with a commercially available nine carbon spacer molecule (Figure 6). This new S-ODN had the sequence 5'-d(GGG~GGG)-3'. When OMA-BL-1 cells were treated with this compound, a decrease in the cell viability was observed when compared with the normal TAG 9-2 S-ODN (Figure 6). Besides the data shown on the graph the middle three bases of TAG 9-1 were also replaced with the nine carbon spacer. This was done to control for any cytotoxic effects that were inherent to the spacer itself. The TAG 9-1 spacer S-ODN did not show any significant effects on the viability of OMA-BL-1 cells. This experiment demonstrates the feasibility of replacing the TTA portion of TAG 9-2 with a 9-carbon spacer.

In addition to the 9-carbon spacer an abasic site spacer was also used to replace the TTA portion of TAG 9-2. The abasic site spacer is a deoxyribose sugar without a nucleotide, but that still has a sulfur atom replacing one of the nonbridging oxygen atoms in the phosphodiester bond, making it a phosphorothioate. We denote the presence of this spacer with a ^ symbol. This spacer was used to determine whether or not the increased efficacy seen with the TAG 9-2~ ODN was due to an intrinsic property of the 9-carbon spacer, or if it was merely the result of the substitution, or any generic spacer. When OMA-BL-1 cells were treated with TAG 9-2^ and TAG 9-2~ it was observed that TAG 9-2^ decreased cell viability more than the TAG 9-2~. (Figure 7) In parallel with this experiment TAG 9-1^ with the three guanine nucleotides in the middle of the molecule replaced by the abasic site spacer was also tested on OMA-BL-1 cells. This S-ODN did not have any effect on the cells. The increase in efficacy observed with the TTA portion of the molecule is replaced with a spacer molecule is not due to any intrinsic effects of a specific spacer, but in fact due to the substitution itself.

Telomerase Repeat Amplification

A series of TRAP (Telomerase Repeat Amplification Protocol) Assays was also performed on all of the S-ODNs that were used in the cell culture experiments. The TRAP assay allows one to detect telomerase activity present

in cell lysates or tissue lysates. The TRAP assays are used to corroborate the data from the cell culture experiments.

When TAG 9-2 was added to cell lysates a complete inhibition of telomerase was seen at all concentrations tested (.3,1,3, and 10). This is in agreement with the results of the cell culture experiments, however, TAG 9-2 is able to inhibit telomerase at concentrations below which it is unable to exert cytotoxic effects on OMA-BL-1 cells.

When TAG 9-1 was tested in this assay system the results also corroborated the cell culture experiments. In cell culture TAG 9-1 does not decrease the viability of OMA-BL-1 cells. In the TRAP assay TAG 9-1 is not able to inhibit telomerase at lower concentrations. At higher concentrations (>10μM) the inhibition of telomerase is likely no longer a sequence specific event so that inhibition of telomerase by TAG 9-1 at high concentrations is not surprising.

The glyoxalated TAG 9-2 was also tested with the TRAP assay. This S-ODN was not able to inhibit telomerase at concentrations up to 10μM. The glyoxalation reaction rendered TAG 9-2 unable to inhibit telomerase. This result would be expected as the glyoxalation reaction would render TAG 9-2 unable to hydrogen bond. In cell culture this S-ODN exhibited a decreased efficacy, in the TRAP assay this compound was not able to inhibit telomerase.

The TAG 9-2 glycerol S-ODN was able to inhibit telomerase at concentrations >3μM. This compound is still able to interact with the telomerase enzyme, but it is not able to receive the hexameric additions that the enzyme catalyzes. While the addition of hexameric repeats onto the S-ODNs does play some role in the mechanism of inhibition of telomere mimetic sequences, the more important aspect of the mechanism is the ability to interact, or hydrogen bond, with the enzyme.

The addition of one guanine nucleotide to the 5' end of TAG 9-2 increased its cytotoxic effects. In the TRAP assay the opposite results were seen, the 5' G TAG 9-2 was not able to inhibit telomerase as effectively as

normal TAG 9-2. This is not surprising as the extra guanine nucleotide is not a part of the telomere sequence, conceivable the extra guanine makes it more difficult for the S-ODN to interact with the enzyme.

The two S-ODNs that have the spacer molecules incorporated within them are both effective at inhibiting the telomerase enzyme. They were able to inhibit it at all concentrations tested. This data shows that replacing the TTA portion of the S-ODN does not alter its ability to inhibit telomerase.

EXAMPLE 2

The question was postulated as to whether single stranded telomers act as a dosimeter for radical stress. The average human cell contains approximately 15 kilobases of telomeric sequences (TTAGGG). These telomeres have a very high content of guanine (50%). Guanine has long been known to be the primary target of radical oxygen intermediates. An interaction between a radical oxygen intermediate and a telomere would generate a short fragment of telomeric DNA. Thus the hypothesis was generated that free radical generators such as peroxide or nitric oxide would result in the generation of a telomere fragment and a shortened telomere. As has been previously demonstrated, telomere mimics inhibits telomerase but this inhibition is not responsible for cytotoxicity. Figure 8 depicts the relationship between cytotoxicity and telomerase inhibition of several of the compounds generated according to the invention.

To test the effects of nitric oxide on telomere mimic cytotoxicity, 50 μM of nitroprusside were added to Oma-BL1 cells in the presence of TAG 9-2 and TAG 9-1. The results are shown in Table 3. As can be seen the presence of nitroprusside, a potent free radical generator, caused a drastic increase in the lethality of the 9-2 oligo.

Table 3
The Effect of Nitric Oxide on Telomere Mimic Cytotoxicity

	TAG 9-2 5'-GGGTTAGGG-3	TAG 9-1 5'-TTAGGGTTA-3'
Control	.198 ± .060	.191 ± .026
50μM Nitroprusside	0.000 ± .004	.208 ± .082

Similarly an assay was conducted of cell viability in the presence of ascorbate a radical scavenger. The presence of ascorbate greatly inhibited the cytotoxic effects of telomere mimetic compounds. The results of the radical scavenger tests are shown in Figure 9. Thus free radical generators potentiate the cytotoxic effects of active telomere mimetic compounds and free radical scavengers are able to inhibit the cytotoxic effects of those compounds. Thus in accordance with the invention the co-administration of telomere mimetic compounds and free radical generators results in a synergistic increase in cytotoxicity.

Without wishing to be bound by any theory, it is postulated that the telomere mimetic compounds and the radical generating compounds interact in some way which ultimately contributes to the enhanced cytotoxicity. Oma-BL1 were incubated for 48 hours with labeled telomere mimetic compounds. The cells were then fixed and mounted on slides and photographed using fluorescence microscopy. Cells that were incubated with an 8-oxo-compound or with oligonucleotide in the presence of a radical generator (sodium nitroprusside) show a distinct punctate pattern. Cells incubated with normal compound without the radical generator exhibited a distinctly different pattern of accumulation.

Free radical generating compounds in the presence of telomere mimetic sequences results in the generation telomere fragments. The purpose of these fragments may be to act as a signal molecule for high levels of oxidative stress. A measurement of DNA present in media after treatment of cells exposed to TAG 9-2 in the presence of sodium nitroprusside demonstrates a time-dependent increase in the presence of DNA as shown in Figure 10. In Figure 11, the effects of incubation with the telomere compound and with no oligonucleotide clearly demonstrate the increase in endogenous fragments present. Figures 10 and 11 were generated according to the following protocol. Briefly ^{3}H-thymidine labeled Oma-BL1 cells plus nitroprusside were counted in a media scintillation counter. A nitron membrane with oligonucleotide cross

linked to it was hybridized with the media at room temperature for 12 hours. The membrane was then counted in a scintillation counter.

What is claimed is:

1. A method of inhibiting proliferation of and/or killing cells characterized by uncontrolled proliferation comprising: contacting said cells with an oligonucleotide, said having a sequence characterized by: a telomere motif XXXGGG, wherein X is a nucleotide base; a guanine content of greater than 50%; and which does not form guanine secondary pairing structures.

2. The method of claim 1 wherein said oligonucleotide comprises a guanine which is capable of hydrogen bonding.

3. The method of claim 1 wherein said oligonucleotide consists of less than twelve nucleotide bases.

4. The method of claim 1 wherein said oligonucleotide comprises a deaza modification to prevent secondary guanine structure formation.

5. The method of claim 1 wherein said oligonucleotide comprises a guanine at its 3' end.

6. The method of claim 1 wherein said oligonucleotide has a guanine at its 5' end.

7. The method of claim 1 wherein said oligonucleotide is free from steric hindrance on the 3' end.

8. A method according to claim 1 wherein said oligonucleotide comprises a guanine adjacent to a guanine triplet motif.

9. The method of claim 1 wherein said cell is characterized by telomerase activity.

10. The method of claim 1 wherein said oligonucleotide is modified to resist enzyme degradation.

11. The method of claim 1 wherein said oligonucleotide has a phosphorothioate backbone modification.

12. The method of claim 1 wherein said oligonucleotide consists of the following nucleotide sequence: GGGXXXGGG wherein X is any nucleotide base.

13. The method of claim 1 wherein said oligonucleotide consists of the following nucleotide sequence: GGGATTGGG.

14. The method of claim 1 wherein said oligonucleotide consists of the following sequence: GGGTTAGGGG.

15. The method of claim 1 further comprising the step of:
contacting said cells with an oxygen radical generator the presence of said oligonucleotide.

16. A method of inhibiting proliferation of and/or killing cells characterized by telomerase expression comprising: contacting said cells with an oligonucleotide, said having a sequence characterized by: a telomere motif XXXGGG, wherein X is a any nucleotide base; a guanine content of greater than 50%; and which does not form guanine secondary pairing structures.

17. The method of claim 16 wherein said oligonucleotide is characterized by a guanine at its 3' end.

18. The method of claim 16 wherein said oligonucleotide has a guanine at its 5' end.

19. The method of claim 16 wherein said oligonucleotide is modified to resist enzyme degradation.

20. The method of claim 16 wherein said oligonucleotide has a phosphorothioate backbone modification.

21. The method of claim 16 wherein said oligonucleotide consists of the following nucleotide sequence: GGGXXXGGG wherein X is any nucleotide base.

22. The method of claim 16 wherein said oligonucleotide consists of the following sequence GGGTTAGGG.

23. The method of claim 16 further comprising the step of:
contacting said cells with an oxygen radical generator the presence of said oligonucleotide.

24. An oligonucleotide composition optimized for inhibition of cell proliferation of and/or killing of neoplastic cells consisting of less than twelve nucleotide bases, said oligonucleotide having a sequence characterized by: a telomere motif XXXGGG wherein X is any nucleotide base; a guanine content of greater than 50%; and a guanine base capable of hydrogen bonding.

25. The composition of claim 24 wherein said oligonucleotide has a guanine at its 3' end.

26. The composition of claim 24 wherein said oligonucleotide has a guanine at its 5' end.

27. The composition of claim 24 wherein said oligonucleotide is free from steric hindrance on the 3' end.

28. A composition according to claim 24 wherein said oligonucleotide comprises a guanine adjacent to a guanine triplet motif.

29. The composition of claim 24 wherein said oligonucleotide is modified to resist enzyme degradation.

30. The composition of claim 24 wherein said oligonucleotide consists of the following nucleotide sequence: GGGXXXGGG wherein X is any nucleotide base.

31. The composition of claim 24 wherein said oligonucleotide has a phosphorothioate backbone modification.

32. The composition of claim 24 wherein said oligonucleotide consists of the following sequence GGGTTAGGG.

33. A pharmaceutical composition for inhibiting diseases characterized by uncontrolled proliferation of cells comprising: a pharmaceutically effective amount of the oligonucleotide of claim 24, and a pharmaceutical carrier.

34. The composition of claim 33 wherein said oligonucleotide has a guanine at its 3' end.

35. The composition of claim 33 wherein said oligonucleotide has a guanine at its 5' end.

36. The composition of claim 33 wherein said oligonucleotide is free from steric hindrance on the 3' end.

37. A composition according to claim 33 wherein said oligonucleotide comprises a guanine adjacent to a guanine triplet motif.

38. The composition of claim 33 wherein said oligonucleotide is modified to resist enzyme degradation.

39. The composition of claim 33 wherein said oligonucleotide has a phosphorothioate backbone modification.

40. The composition of claim 33 wherein said oligonucleotide consists of the following nucleotide sequence: GGGXXXGGG wherein X is any nucleotide base.

41. The composition of claim 33 wherein said oligonucleotide consists of the following sequence GGGTTAGGG.

42. A composition for inhibiting proliferation of and/or killing neoplastic cells comprising: an oligonucleotide comprising a telomere motif, XXXGGG wherein X is a nucleotide; a guanine content of greater than 50%; and a

guanine base capable of hydrogen bonding; said oligonucleotide being one which consists of less than twelve nucleotide bases.

43. The composition of claim 42 wherein said oligonucleotide has a guanine at its 3' end.

44. The composition of claim 42 wherein said oligonucleotide has a guanine at its 5' end.

45. The composition of claim 42 wherein said oligonucleotide is free from steric hindrance on the 3' end.

46. A composition according to claim 42 wherein said oligonucleotide comprises a guanine adjacent to a guanine triplet motif.

47. The composition of claim 42 wherein said oligonucleotide is modified to resist enzyme degradation.

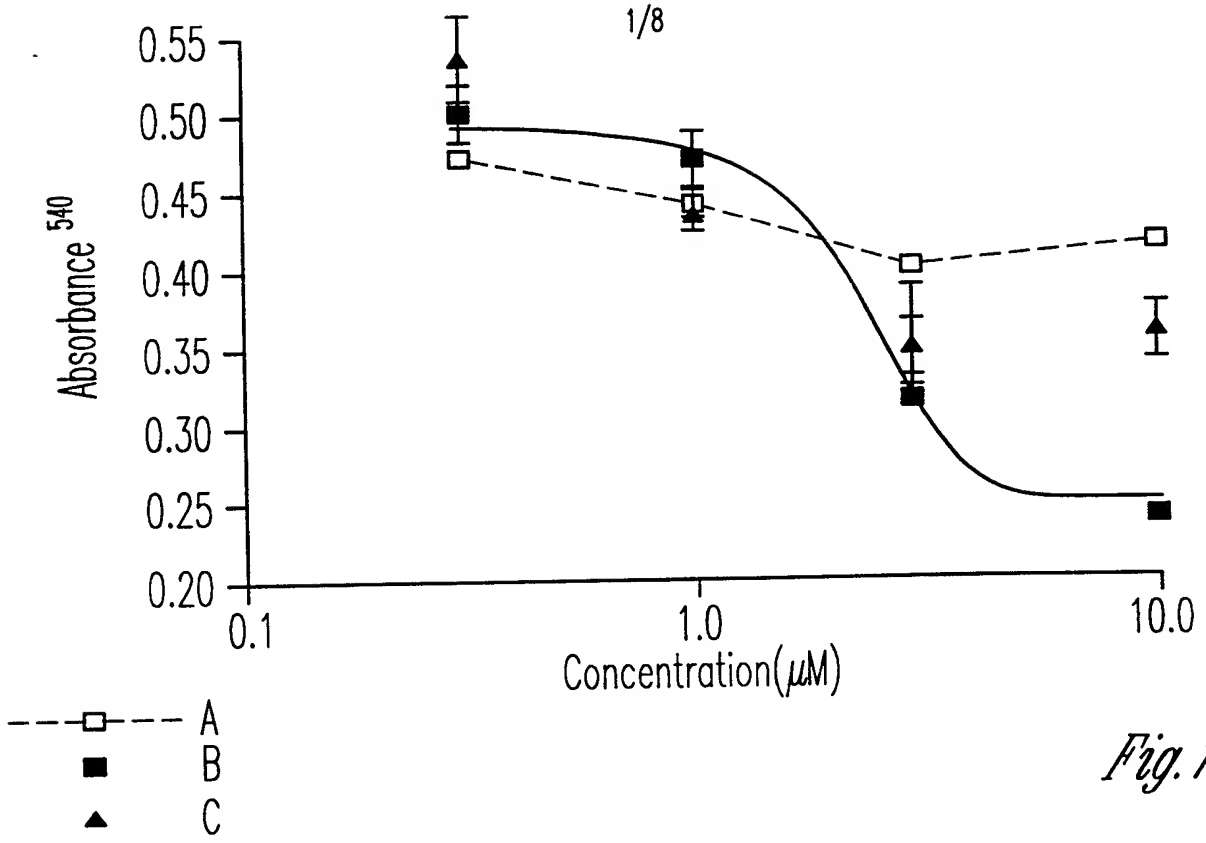
48. The composition of claim 42 wherein said oligonucleotide has a phosphorothioate backbone modification.

49. A method of inhibiting cell proliferation and/or killing neoplastic cells comprising: administering to said cells an effective amount of an telomere mimetic oligonucleotide which has been optimized for cellular uptake, cytotoxicity and telomerase inhibition wherein said oligonucleotide sequence comprises at least two guanine triplets joined by a non-nucleotide spacer molecule so that said triplets are capable of interacting with a telomere motif.

50. A method for enhancing the cytotoxicity of an oligonucleotide comprising multiple telomere repeats, $(TTAGGG)_x$ comprising: modifying said oligonucleotide to prevent secondary structure guanine pairing.

51. The method of claim 50 wherein said modification is accomplished by forming a 7-deaza-G incorporation.

52. An oligonucleotide composition optimized for inhibition of cell proliferation of and/or killing of neoplastic cells said oligonucleotide comprising: more than one telomere motif repeat, XXXGGG wherein X is a nucleotide base said oligonucleotide including a modification to prevent secondary structure formation by guanine pairings.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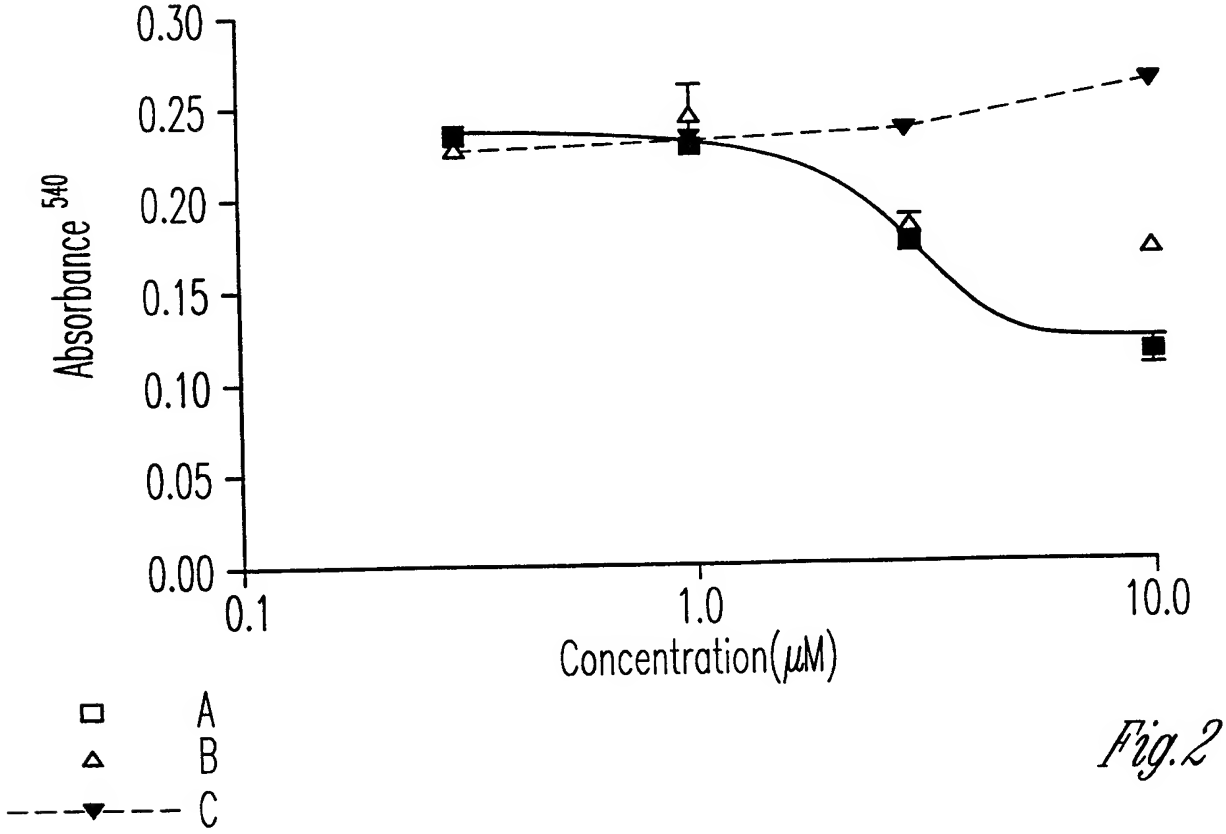
10.0
Concentration(μM)
A
B
C

Fig. 1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B
C

Fig. 2

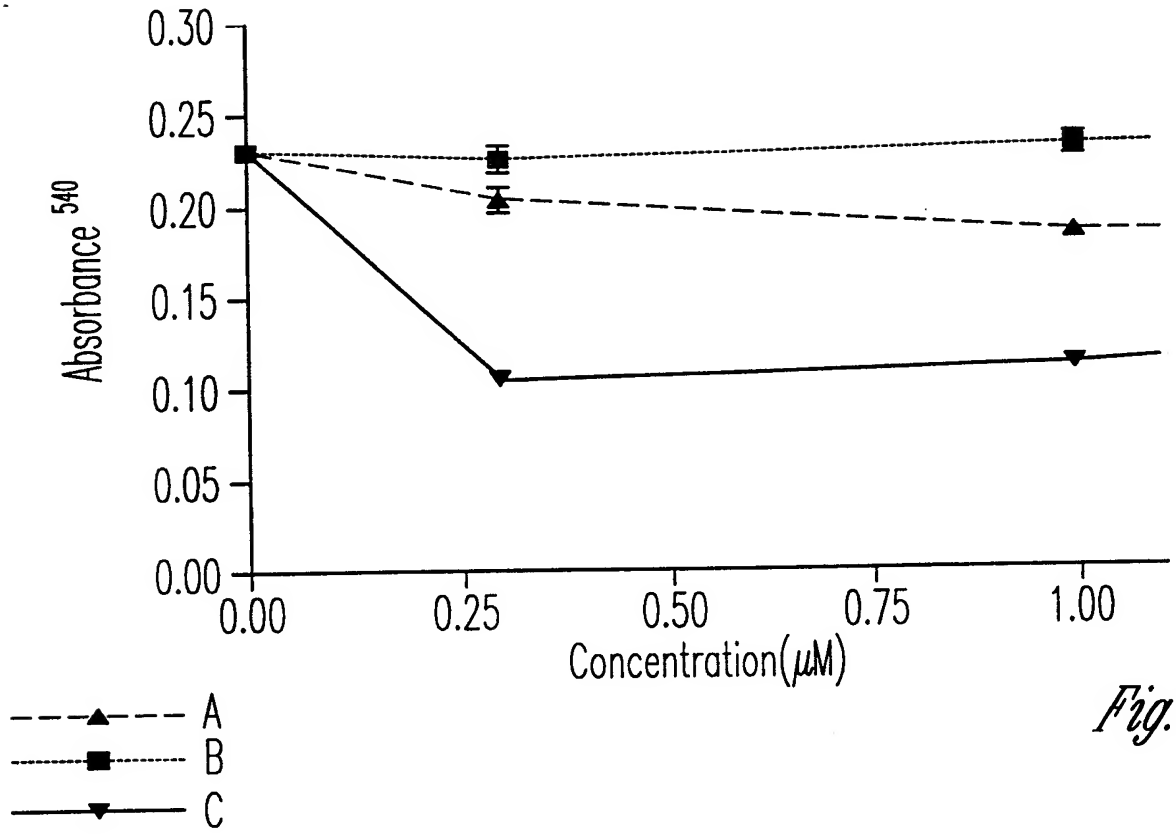
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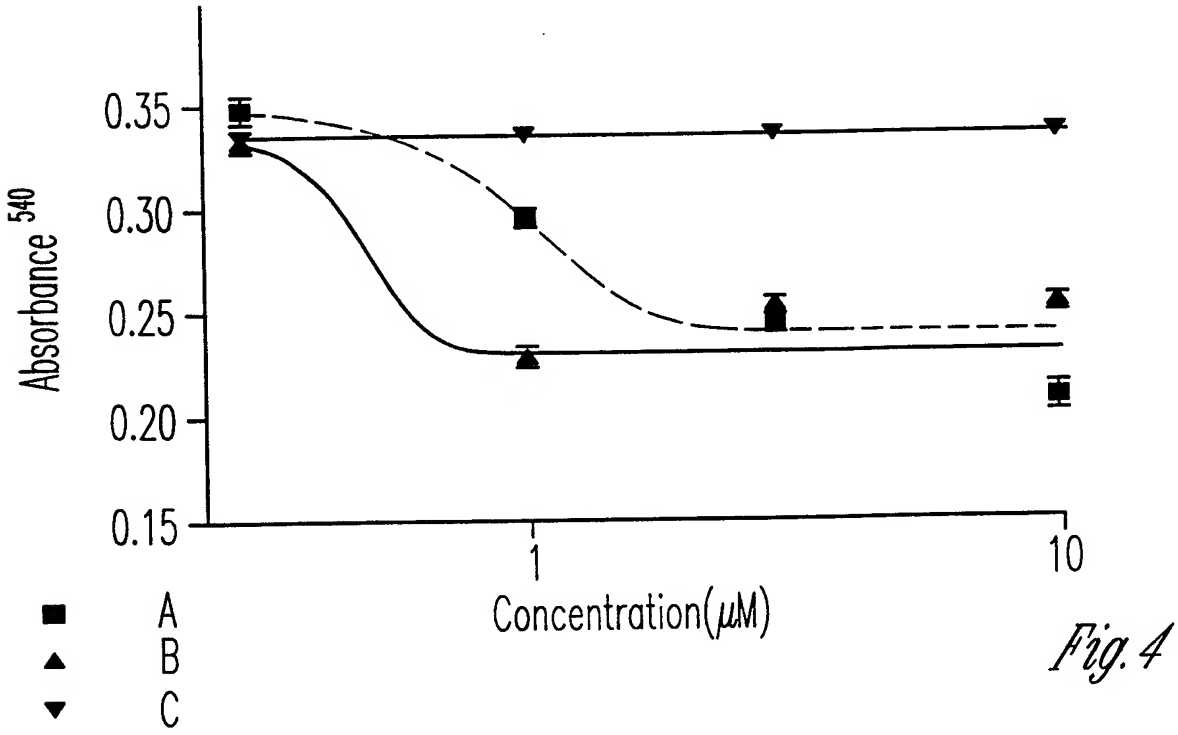
Fig. 3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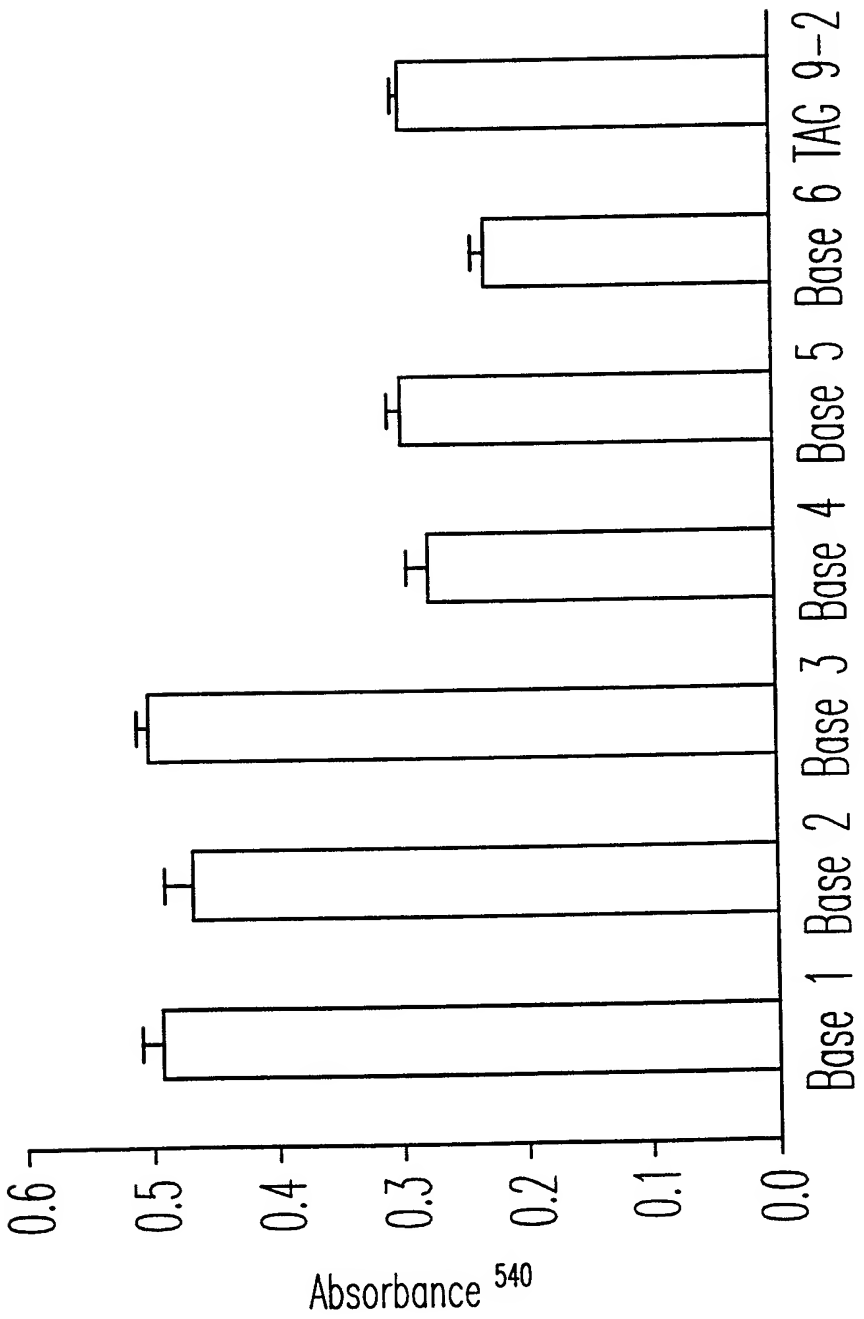
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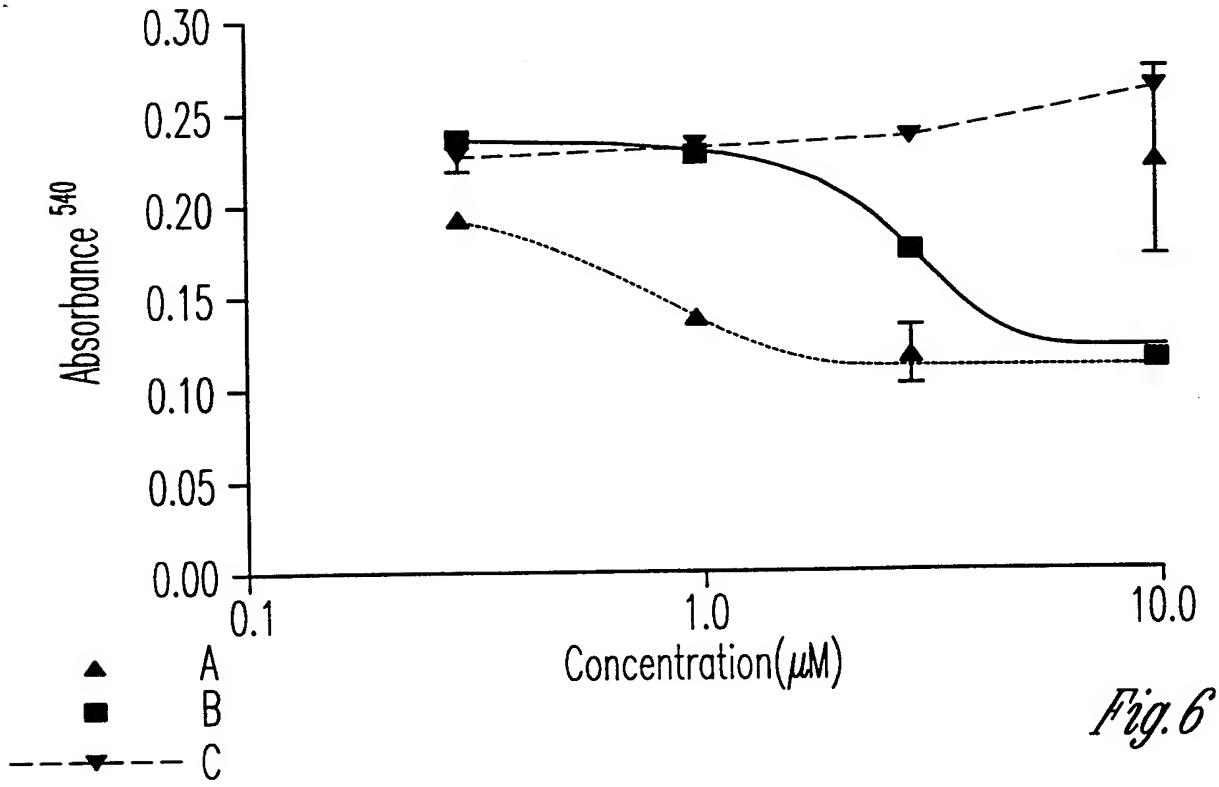
TAG 9-2

Fig.5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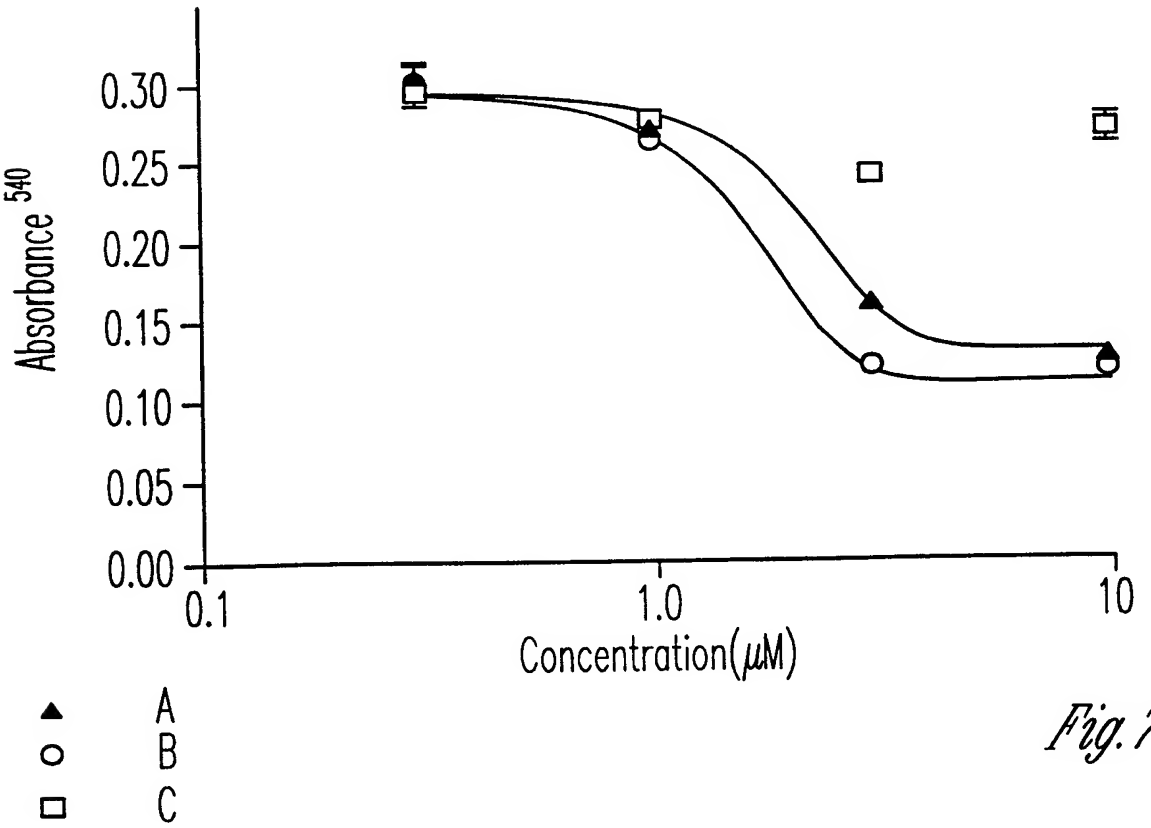
Concentration(μM)
A
B
C

Fig. 6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Concentration(μM)
A
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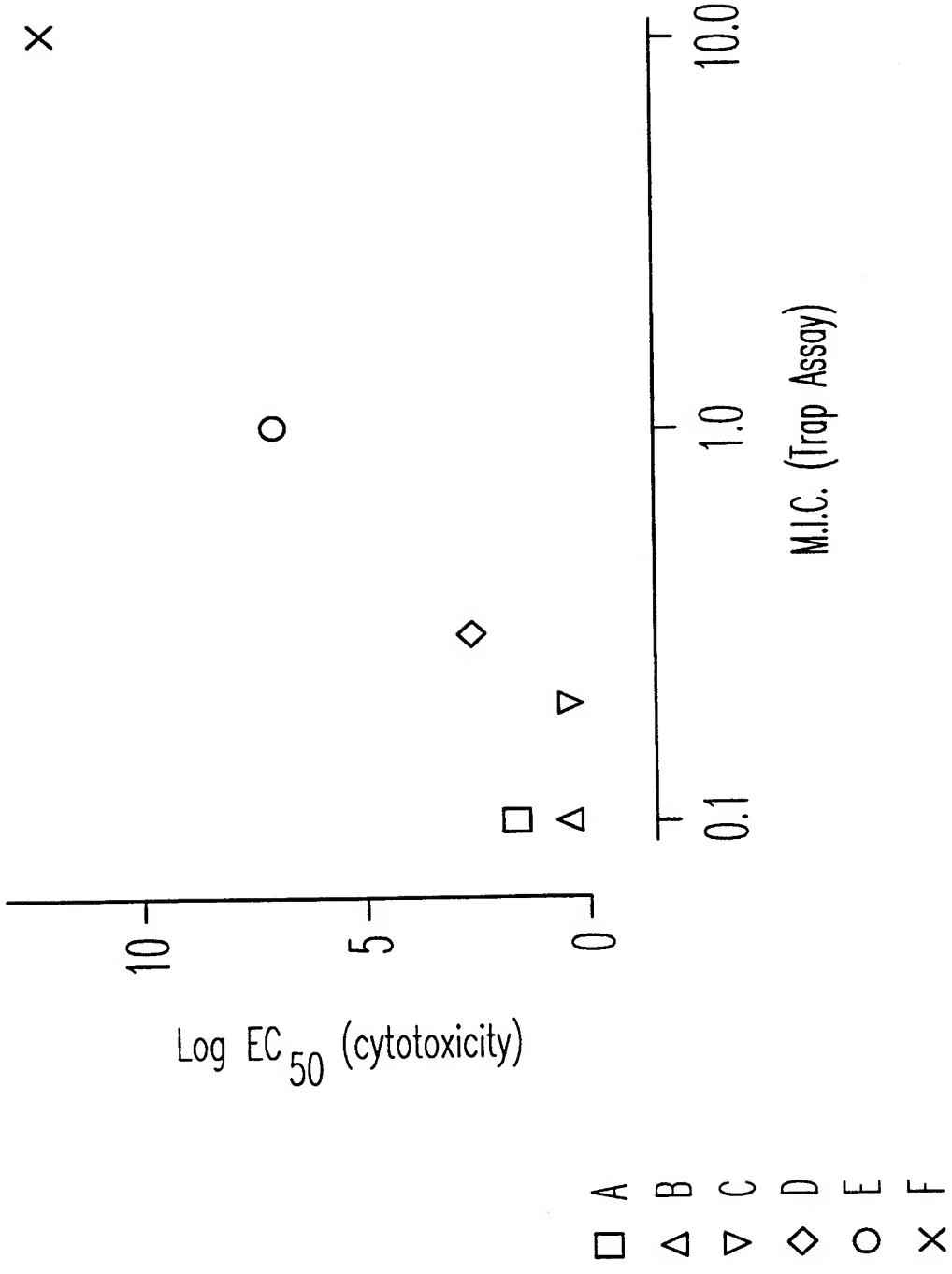
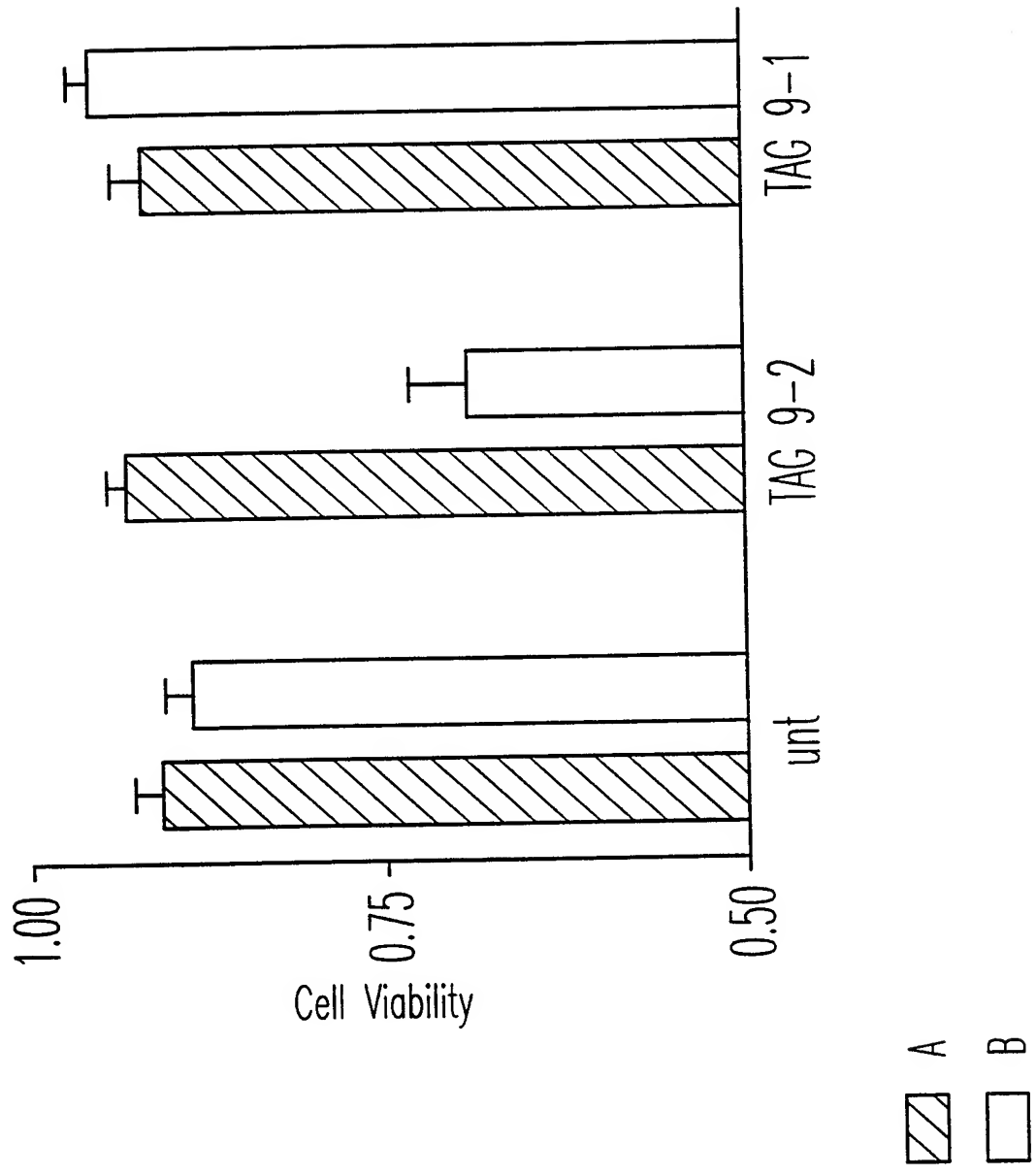
Fig. 7

Fig.8





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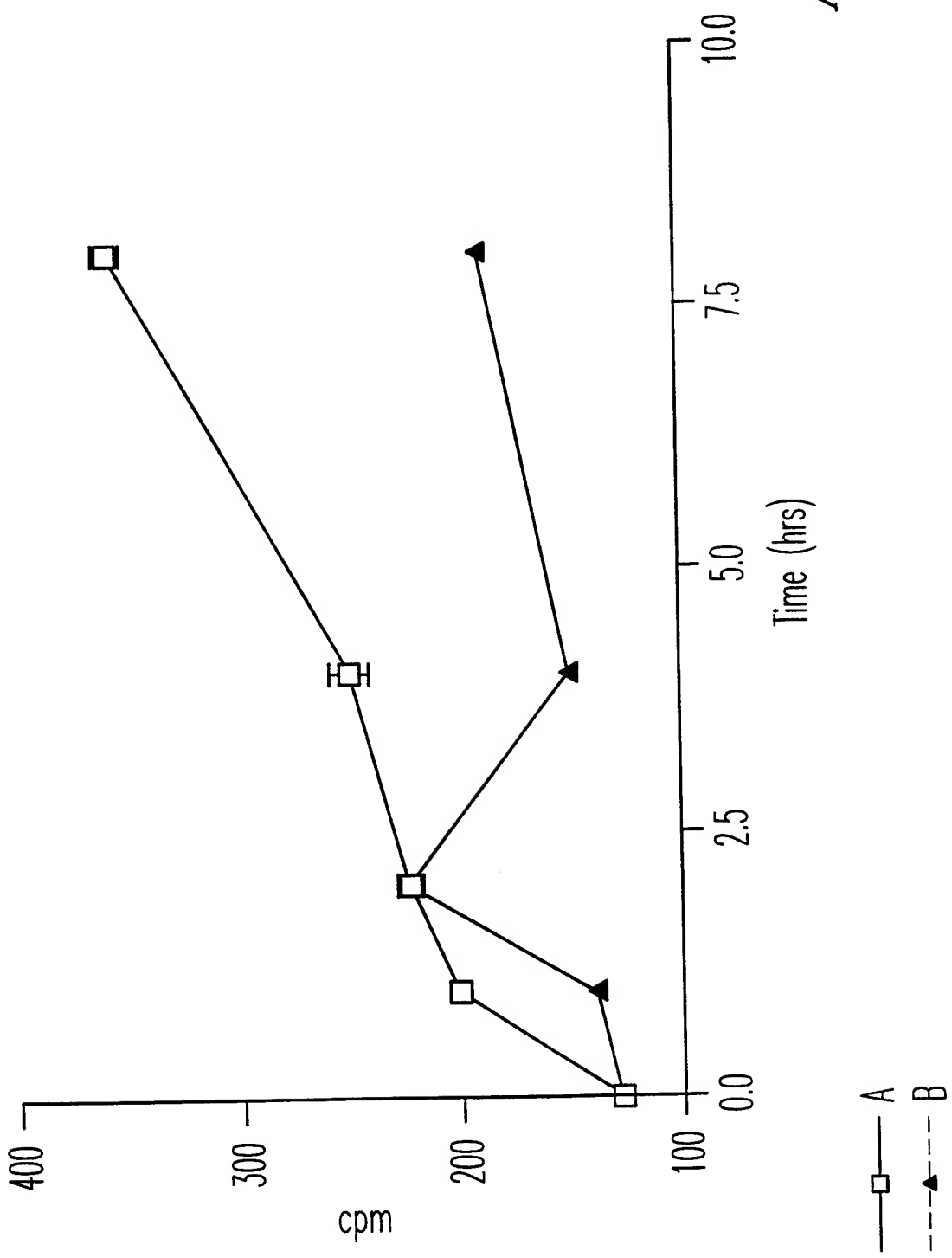

Fig. 10

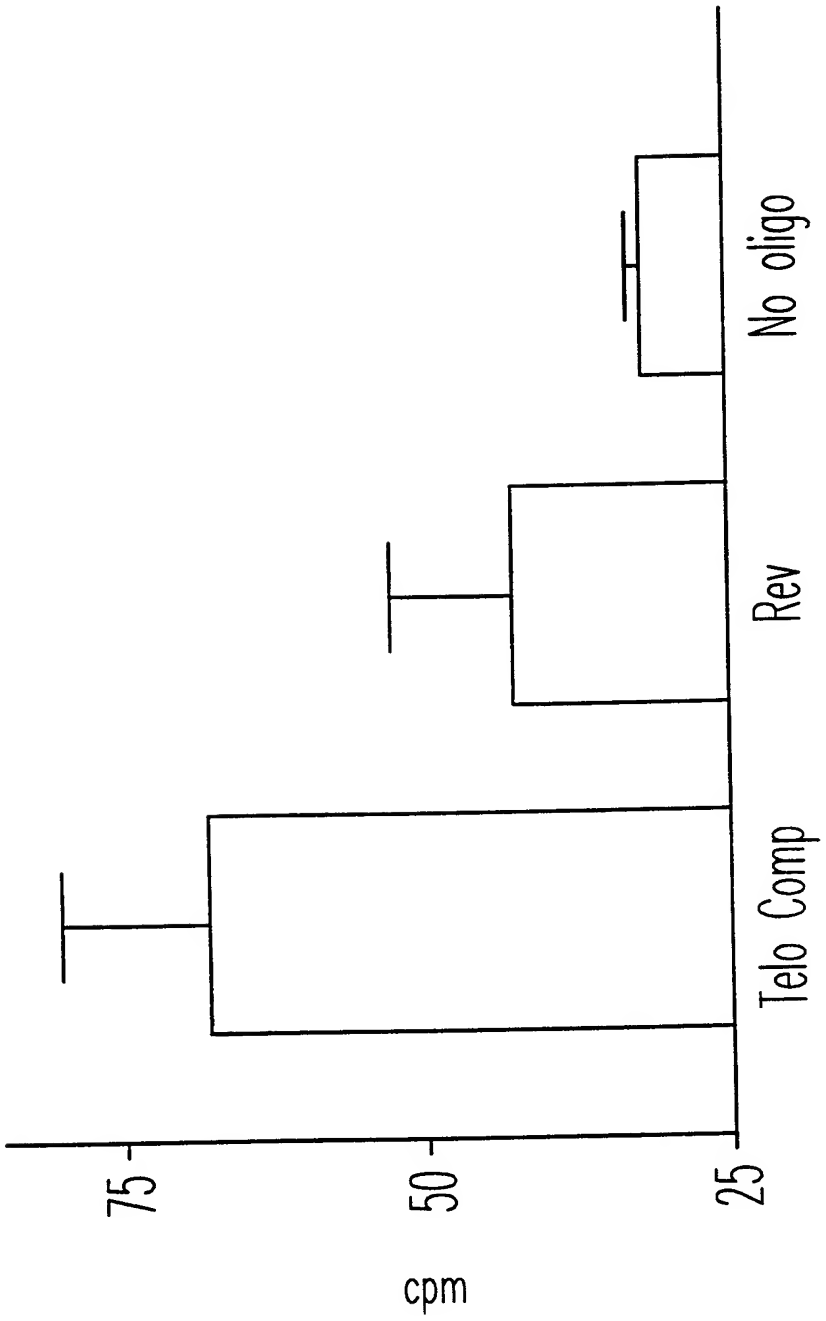

Fig. 11

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14763

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 5/10; C07H 21/04
US CL :514/44; 424/93.21; 435/375; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21; 435/375; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE BIOSIS EMBASE CAPLUS SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,643,890 A (IVERSEN et al) 01 July 1997, see entire document.	1-3, 5, 6, 8-11, 16-20, 24-29, 31, 34-39, 42-48 --- 4, 7, 12-15, 21-23, 30, 32, 40, 41, 49-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 OCTOBER 1998	Date of mailing of the international search report 30 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SEAN MCGARRY for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14763

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	MATA et al. A Hexameric Phosphorothioate Oligonucleotide Telomerase Inhibitor Arrests Growth of Burkitt's Lymphoma Cells in Vitro and in Vivo. Toxicology and Applied Pharmacolgy. May 1997, Vol. 144, pages 189-197, see entire document.	1-3, 5, 6, 8-11, 16-20, 24-29, 31, 34-39, 42-48 ------- 4, 7, 12-15, 21-23, 30, 32, 40-41, 49-52
Y	ZAHLER et al. Inhibition of Telomerase by G-Quartet DNA Structures. Nature. 25 April 1991, Vol. 350, pages 718-720, see entire document.	1-52
Y	FLETCHER et al. Human Telomerase Inhibition by 7-Deaza-2'deoxypurine Nucleoside Triphosphates. Biochemistry. 10 December 1996, Vol. 35, No. 49, pages 15611-15617, see entire document.	1-52